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PRINCIPAL INVESTIGATOR: Dr. Keith R. Duncan

CONTRACTING ORGANIZATION: Regents of the University of Michigan
Ann Arbor, MI 48109

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Dr. Robert Duncan, PhD

E-Mail: rkduncan@umich.edu

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Regents of The University of Michigan
1058 Wolverine Tower
3003 South State Street
Ann Arbor, Michigan 48109-1274

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14. ABSTRACT

The chief aim of our study is restoration of hearing by regeneration of peripheral auditory neurons. The study takes a systematic approach in three objectives aiming to push human stem cells toward an auditory neural fate, embed the cells on a functionalized scaffold, and implant the device in a deafened animal model. In the first year of the project grant, we have addressed three key tasks: (1) derivation of sensory neurons from human pluripotent stem cells (hPSCs), (2) development of implantable nanofibrous substrates, and (3) optimization of the deafness model. We have established and compared several methods for generating glutamatergic sensory neurons including (i) standard SMAD inhibition, (ii) Neurog1-overexpression in otic precursors, and (iii) neurogenesis in inner ear organoids. Neural progenitors adhere and mature on nanofibrous scaffolds. New methods were developed to bundle cell-seeded scaffolds in small, defined polymer sheaths. Surgical approaches were generated to facilitate implantation in chemically deafened guinea pigs. Initial results show improved electrically-evoked auditory brainstem responses in cell-seeded implants compared to control, cell-free implants.

15. SUBJECT TERMS
Human stem cells, neurodifferentiation, deafness model, nanofiber

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I. INTRODUCTION:

An estimated 300 million individuals world-wide suffer some form of hearing loss, and military personnel exhibit auditory deficits at an exceptionally disproportionate level compared with the lay population. In fact, the ear is the organ most affected by concussive or penetrating injuries to the head. The devastating impact of widespread hearing loss on practical, social, and economic levels drives our interest in devising ways to restore hearing completely. Complete restoration of hearing is a particularly challenging task. In this project, we explore a systematic approach for developing a next-generation auditory prosthesis by replacing damaged auditory nerve with stem cell-derived neurons, grown on a nanofiber substrate, and electrically stimulated. We have divided our research goals into three primary objections. Objective 1 seeks to recapitulate the physiology of auditory neurons using human pluripotent stem cells. In Objective 2, we will develop a mat of aligned nanofibers to guide the arrangement of stem cell-derived neurons to mimic the arrangement as well as the physiology of the auditory ganglion. We also develop the means to electrically stimulate derived neurons on this nanofiber substrate. And finally, in Objective 3, we will implant a prototype device in an animal model of neural hearing loss, examining the capacity for stem cell-derived neurons to functionally integrate with the auditory brainstem.

Keywords:
Auditory Neurons, Human Pluripotent Stem Cells, Nanofibrous Substrate, Neural Hearing Loss

ESCs - embryonic stem cells
EBs - embryoid bodies
SMADi - SMAD-inhibitors
RA - retinoic acid
Pur - purmorphamine
IAM - internal auditory meatus

II. BODY:

A. KEY RESEARCH ACCOMPLISHMENTS

Tasks and deliverables are organized according to the major Objectives in the proposal. Progress on Year 1 tasks is bulleted along with any challenges and recommendations. In some cases, tasks originally scheduled for Years 2-4 were initiated early, and progress is bulleted accordingly. All other tasks are listed as “pending”.

Task 1. IACUC review of animal care and use application. The application is under development and will be submitted prior to any award. We estimate some time for modification of this protocol based on feedback from reviewers

- Complete

Objective 1: Evaluate the combination of genetic and neurotrophic cues on the differentiation of adult hiPSCs toward an auditory nerve-like phenotype

Task 2-5. Gene expression analysis using RNA sequencing to compare the transcriptomes of induced cells and native auditory nerve, upregulation of neurotrophic factor receptors and BDNF/NT3-effects on electrical properties.

- Our goal in Objective 1 is to establish a reliable, efficient method for pushing embryonic stem cells (ESCs) to an auditory-nerve fate. In fact, this is an essential part of every objective. We have taken three separate approaches, outlined below.
- Approach 1: This method, reported in prior Progress Reports and based on Kim et al. (2011), is a reliable method for generating neural progenitors that differentiate into glutamatergic sensory neurons. Briefly, embryoid bodies (EBs) are generated by spin-aggregation in a micropatterned plate and pushed toward a neuroectodermal fate using SMAD-inhibitors (SMADi) noggin and dorsomorphin. On day 2, EBs are plated onto Matrigel and incubated in DMEM/F12 with retinoic acid (RA) and purmorphamine

(Pur). We completed a gene expression profile of the influence of RA and Pur on major neural induction genes (bHLH transcription factors) and patterning transcription factors (Pax, Six, Eya). While Pur should produce more ventral fates (switch in Pax2 for Pax6-expressing placode-like sensory neurons), we found high expression of both factors in our cultures. Moreover, the SMAD-inhibitor method produced neurons driven by the bHLH gene Ascl1 rather than otic-inducing Neurog1. As a result, we continue to pursue alternative methods for generating otic like neurons while we continue with later objectives using the SMADi protocol. See Year 2 Progress Report for more information on the derived phenotype. Importantly, we have replicated Approach 1 with the human ESC cell line H9.CreLoxGFP, which will allow us to trace our cells in implants using constitutive, stable GFP expression. To improve detectability of the GFP, we will use an antibody directed against the hrGFP expressed by these cells. We have confirmed the ability to detect GFP in terminally differentiated neurons in cell culture, and we are evaluating trial implants now to test the stability of GFP after implantation.

- Approach 2: In the Year 2 Progress Report, we described the initial steps made toward deriving preplacodal cells based on a protocol by Leung et al. (2013). The goal here is to pre-differentiate to a preplacodal fate and then drive the otic bHLH gene Neurog1 by viral transduction. Initial experiments with a lentiviral construct showed low transduction efficiency and high toxicity in our human ESCs. Hence, we continue to pursue overexpression of Neurog1 by Adenovirus (Ad.RGD.EF1alpha.IRES.GFP). We are comparing the overexpression of Neurog1 in human ESCs with overexpression in cells predifferentiated to a sensory placode fate. To predifferentiate the cells, we adapted the protocol by Leung et al. (2013) as described in the Year 2 report (pilot data). As described previously, we found the Leung et al. protocol (Figure 1A) to generate heterogeneous cultures with poor reproducibility in the expression of the otic marker Six1. BMP activity is essential for formation of the otic preplacode, with a steep gradient in BMP shifting cell fate from non-neural ectoderm (epidermis) to neural plate (Figure 1B). Placodes are established with small amounts of BMP, suggesting that this morphogen is extremely important and able to tip the balance between many cell fates. We tested and found endogenous BMP expression in our cells, indicating that autocrine control could be a major contributor to the heterogeneity in our cultures (Figure 1C). Indeed, the Leung et al. protocol produced otic gene expression like Six1 and Gata3 but also marked expression of the epidermal marker TP63 (Figure 1D). Therefore, we sought to manipulate BMP signaling in order to limit high-BMP fates (epidermis) and BMP-inhibited fates (neural plate). Moderate inhibition of BMP signaling with low concentration of LDN212854 was effective in reducing TP63 while still maintaining Six1 and Gata3 expression. Additionally, BMP inhibition enhances the expression of the otic marker Pax2 (Figure 1D-F).
- Approach 2, continued: Neurog1 is a neuronal inducer involved in differentiation of auditory neurons (Ma et al., 2000). We are thus examining if overexpression in predifferentiated cells can enhance auditory specificity. Overexpression of the adenoviral vector Ad.RGD.Ef1alpha.Neurog1.IRES.GFP induced neural marker expression (Nestin, TUJ1) both in human ESCs and LDN20-induced placodal cells (Figure 2). Transduction efficiency is high in both cell types (>80%). We are currently comparing the efficiency of neurodifferentiation and the expression of auditory markers derived by this approach. Ultimately, evidence of a more auditory-like phenotype will cause us to apply these cells to experiments in Objective 2 and 3, in place of Approach 1.
- Approach 3: In the last 2 years, a new method for generating auditory cell types from stem cells arose. Dr. Eri Hashino established a stepwise program for creating inner ear “organoids” from mouse embryonic stem cells (Koehler and Hashino, 2014). We have successfully used the inner ear organoid method to drive auditory differentiation of mouse ESCs (Figure 3A) using a Pax2-reporter cell line. At day 0, mouse ESCs are aggregated into embryoid bodies using round-bottom 96-well plates. On day 1, growth factor-reduced Matrigel is added at a final concentration of 2% v/v. On day 3, BMP4 (10 ng/mL) and a TGFβ pathway inhibitor (SB431542, 1μM) are added to restrict formation of mesoderm and endoderm and to promote non-neural ectoderm differentiation. On day 4.5, FGF2 (100 ng/mL) and a BMP receptor inhibitor (LDN193189, 1μM) are added to promote otic placodal differentiaton. Otic vesicle-like structures to form after the aggregates are transferred to a basic maturation medium based on experiments with $Pax2^{EGFP/+}$ ESCs, in which these structures are visible via epifluorescence. Over the next several days, these vesicles expand and push to the aggregates’ outer surfaces. By day 20, we detect innervated hair cells at each organoid, i.e., the interface between the expanded, protruding vesicle and the rest of the aggregate (Figure 3B,C) . Immunofluorescence staining shows that neurons

are abundant near the hair cells, and neural projections pass through and terminate at the hair cell layer (Figure 3C-E). Staining for Ctbp2, a characteristic marker of the presynaptic ribbon synapse in hair cells, reveals multiple ribbon synapses per Myo7a^{+} hair cell at day 24. Our preliminary studies were performed with Pax2$^{EGFP/+}$ ESCs to allow visualization of developing vesicles. However, to visualize stem cell-derived neurons, we will use the TK23 ESC line expressing GFP under the promoter for endogenous tau, a microtubule-associated protein. This procedure will allow us to cell sort GFP+ neurons and use these in place of those derived in the above approaches. This is an alternative approach but adds considerable value since we can confirm a truly inner ear origin. In parallel experiments, we are collaborating with Dr. Michael Roberts to show integration of these neurons with the auditory brainstem. Again, positive results would cause us to focus attention on this cell source.

Objective 2: Design a first-generation electrical interface for stimulation of stem cell-derived neurons grown on a nanofiber substrate. All tasks rely solely on cell lines.

Task 6. Minimize thickness of nanofiber mat to maximize flexibility

- Complete

Tasks 7-12. Design, implement, and examine efficacy of electrical interface to nanofiber substrate

- The phenotype of human ESC-derived neurons on nanofiber mats has been evaluated. Neural progenitors from Approach 1 (see Objective 1) were grown on polystyrene coverslips as well as unaligned and aligned nanofiber mats. After 4 weeks of differentiation, the progenitors adopt a neural fate with high efficiency, retain a glutamatergic phenotype, express mature neuron markers like Map2, and are capable of forming synaptic contacts with Synaptophysin (Figure 4). The nanofibers appeared to limit proliferation (fewer neurons) particularly the unaligned configuration. Aligned fibers tended to produce neurons with a bipolar morphology, which is desirable for auditory nerve replacement. These preparations are being further evaluated for fiber-influence on glial fate, proliferation, and other neurotransmitter systems. When the neurons were grown on coverslips, they tended to fasciculate into bundled projections giving some appearance of alignment, but the bundling was randomly oriented overall. When grown on unaligned nanofibers, the fasciculation was prevented and there was no evidence of alignment or patterning. In contrast, the aligned nanofibers effectively aligned neurite projections (see Figure 5).
- Pending task: integrate with electrode array. This has been hampered by the reduced size of the scaffold. Original plans to integrate with a high-density paralene array require a larger diameter (less curvature) of the scaffold's conduit, but the IAM of the guinea pig and limits imposed by the surgical approach make this difficult. Alternatives are being pursued, including a linear array place within the scaffold. In the meantime, we have implanted scaffolds with a single electrode in Objective 3.
- Pending task: The calcium imaging system has been setup and will be used to determine activity in cell-seeded scaffolds, and the degree to which a linear electrode array can stimulate individual groups of neurons.

Objective 3: Optimize the integration of stem cell-derived neurons following in vivo transplantation of the seeded nanofibrous scaffold

Task 13. Group 1: Pilot deafening. Confirm efficacy of ß-bungarotoxin in guinea pig and time point of nerve death. 10 guinea pigs will be required, including an expected failure rate of 30% from morbidity and mortality

- See Year 1 report; ouabain treatment produces the desirable neuron loss in guinea pigs.

Task 14. Group 2: Pilot implantation. Confirm positional stability and integrity of implant over time. 15 guinea pigs will be required, including an expected failure rate of 30% from morbidity and mortality. No hiPSCs involved

- While advancing the challenging experiments in Objectives 1 and 2, we have spent considerable attention on Objective. We are making major progress with the first implantable aligned auditory nerve graft.
- We optimized the surgical approach and have defined the implant design constraints. Polymer blends of poly-lactic acid and poly-caprolactone were found to be best for cell adhesion, fiber dimensions, reproducibility, and stability over time. A method for producing poly-caprolactone sheaths was established to achieve the necessary stiffness and stability for implantation. Moreover, methods for wicking fiber bundles into the sheaths were established so that we could cell neural progenitors onto exposed fiber bundles, then shift the sheath overtop of the adherent cells, cut the conduit away, and prepare for implantation (Figure 6 A-C). About 1,000-2,500 cells are seeded onto the scaffolds (Figure 6D) with evidence of TUJ1-positive neurite extension along the fibers even 1 day after seeding (Figure 6E). We found adhesion was gradually lost over several weeks, but that adhesion was unchanged after 1 day (Figure 6F). Therefore, we implant 1 day after cell seeding. In some cultures, with minimal disturbance during 6 weeks of media changes (about 20 times), we could find evidence of aligned neurites extended the length of the fiber bundle (Figure 6G). Therefore, we concluded that the loss of cells during culture was not an issue of survival but due to adhesion problems in cultures that were constantly perturbed. Now, we can routinely implant conduit scaffolds 0.5-0.7 mm in diameter and 2-2.5 mm long to pass through the cochleostomy and reach the internal auditory meatus (IAM), the passageway linking the auditory nerve ganglion with the auditory brainstem. See Year 1 and Year 2 Reports for methods/results on electrospinning the nanofibers and forming the conduit.
- Surgical approach: We used guinea pig temporal bones to develop the surgical approach for implantation within the internal auditory meatus (IAM), as described in the Year 2 report. Several animal subjects were evaluated using sham operations (deafening and scaffold insertion only, no persistent implanted device) and others were implanted with cell-free conduits. These were used to determine any adverse tissue reaction and evidence of tissue repair. The data are summarized in Figure 7. Nanofibrous scaffolds are easily sectioned and show minimal evidence of scarring (Figure 7A). However, endogenous cells infiltrate the full length of the scaffold (Figure 7B). We used immunohistochemistry to evaluate this further. We found no evidence of persistent inflammatory responses 4 weeks after implantation (CD45-negative) (Figure 7C). Vestibular neurons were identified outside the scaffold area on the border of the IAM. Both Schwann cells and astroglia were identified in the IAM after surgery but only Schwann cells significantly migrated into the scaffolds. These cells may provide additional trophic support for neural progenitors on cell-seeded scaffolds. This possibility is being evaluated in experiments already underway.

Tasks 15 through 18 examine the functional integration of hiPSC-derived neurons following implantation of the neuralized nanofibers.

- Functional recovery (Figure 8). Thresholds are significantly better 10 days after implantation with H9 neural progenitors compared to control subjects receiving cell-free conduits. We found decreases in threshold over time after implantation, regardless of whether the scaffold contained cells or not. Therefore, some of the ABR response is from endogenous neurons. Given the presence of TUJ1-positive neurons in the IAM (Figure 7), possibly from vestibular nerve, some recovery may be due to tissue repair following implantation in neurons other than the auditory nerve or the xenografted cells. More subjects, control and cell-seeded, are currently underway. Correlated histological data will help determine whether the improved thresholds are likely due to the presence and integration of the implanted neural progenitors.
- Parallel implants of cells-alone (no nanofibers) are also underway to determine whether the nanofiber scaffold assists with integration of the exogenous cells.

B. REPORTABLE OUTCOMES

Abstracts

1. Hackelberg, S., Rastogi, A., Tuck, S.J., White, C., Liu, L., Prieskorn, D., Corey, J.M., Miller, J., **Duncan, R.K.** (2015) Nanofibrous scaffolds for the integration of human embryonic stem cells into the cochlea. Abstracts of the Association for Research in Otolaryngology, 38:9.
2. Hackelberg, S., Rastogi, A., Tuck, S.J., White, C., Liu, L., Prieskorn, D., Corey, J.M., Miller, J., **Duncan, R.K.** (2015) A nanofiber guided approach for the integration of human neural precursors into the cochlea. Society for Neuroscience, 31:13.
3. Hackelberg, S., Tuck, S., Rastogi, A., White, C., Liu, L., Prieskorn, D., Miller, J., **Duncan, R.K.**, Corey, J. (2015) Nanofiber scaffolds with integrated neuronal progenitors for the re-engineering of the auditory nerve. Abstracts of the Biomedical Engineering Society (BMES) Annual Meeting, accepted.
4. Hackelberg, S., Tuck, S.J., Rastogi, A., White, C., Liu, L., Prieskorn, D.M., Miller, R., Deremer, S., Chan, C., Corey, J.M., Miller, J.M., **Duncan, R.K.** (2016) A nanofiber guided approach for cochlear SGN regeneration with human neural precursors. Abstracts of the Association for Research in Otolaryngology, 39:accepted.
5. Schaefer, S., **Duncan, R.K.** (2016) Derivation of inner ear organoids from Pax2$^{EGFP/+}$ mouse embryonic stem cells via optimized FGF signaling. Abstracts of the Association for Research in Otolaryngology, 39:accepted.
6. Lee, M.Y. Hackelberg, S., Green, C.L., Lunghamer, K.G., Kurioka, T., **Duncan, R.K.**, Raphael, Y. (2016) Transplanted H9-GFP stem cells survive in scala media of conditioned guinea pig cochlea. Abstracts of the Association for Research in Otolaryngology, 39:accepted.

Grant Application Submission:
VA RR&D Merit Review 10/01/2015-9/30/2017 $200,000
Cell-integrated microfibers for improved nerve regeneration
P.I.: Corey JM

Databases

We have generated a normative database of the number spiral ganglion neurons in the normal and ouabain treated guinea pig. These normative data will form the reference for determining the extent of new neural growth in the implanted deafened ear.

C. CONCLUSION

The research completed to date shows that simple overexpression of Neurog1 is a less efficient method for generating stem cell-derived neurons using hESCs compared with prior work in mESCs. This could reflect basic differences between mouse and human or simple differences in two separate stem cell lines. Regardless, a more robust method for generating auditory nerve-like cells is required, particularly one that can be easily translated to a variety of human pluripotent stem cell lines. We have two viable methods and seek to streamline the stepwise protocol to enhance the speed of differentiation while creating neurons with molecular and physiological phenotypes indicative of auditory neurons.

Our advanced studies on nanofiber scaffold design have provided a unique and novel method for self-rolling nanofibrous substrates. Now, we show improved methods for increasing these stiffness of these substrates with a PCL tube and novel methods for loading the scaffolds with NPCs. Cells are able to survive the process and adhere to the scaffold. Preliminary data shows good alignment of axonal projections along the nanofibers inside the conduits. With improvements in the implantation approach, we are now ready to enter the in vivo phase with cell-seeded conduits, paving the way for a viable auditory nerve graft.

D. REFERENCES

Koehler KR, Hashino E (2014) 3D mouse embryonic stem cell culture for generating inner ear organoids. Nat Protoc 9:1229-1244.

Kim JE, O'Sullivan ML, Sanchez CA, Hwang M, Israel MA, Brennand K, Deerinck TJ, Goldstein LS, Gage FH, Ellisman MH, Ghosh A (2011) Investigating synapse formation and function using human pluripotent stem cell-derived neurons. Proc Natl Acad Sci U S A 108:3005-3010.

Leung AW, Morest DK, Li JYH (2013) Differential BMP signaling controls formation and differentiation of multipotent preplacodal ectoderm progenitors from human embryonic stem cells. Dev Biol 379:208-220.

Ma Q, Anderson DJ, Fritzsch B (2000) Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation. J Assoc Res Otolaryngol 1:129-143.

SUPPORTING DATA

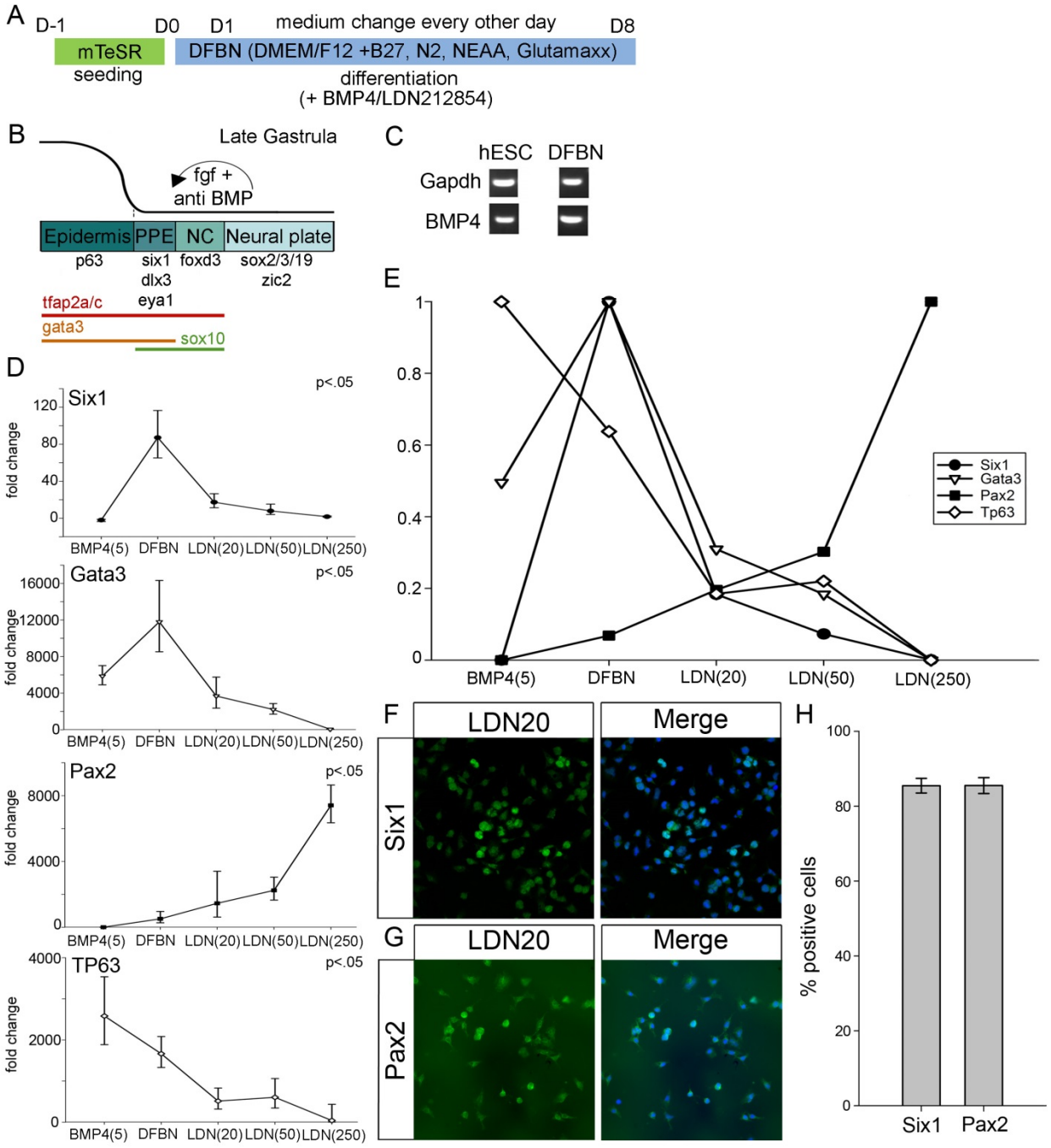

Figure 1. Otic preplacode differentiation protocol. (A) Cartoon of the general differentiation scheme. At day -1 hESCs are dissociated and plated as single cells. The next day (day 0) medium is changed to DFBN medium, a serum free medium based on DMEM/F12. Medium is changed every other day for the remainder of the differentiation protocol. To test for the influence of BMP signaling on the expression of genes that characterize an otic preplacode fate, medium was supplemented either with the BMP agonist BMP4 or the BMP signaling inhibitor LDN212854. After 8 days of differentiation cells were harvested and analyzed with standard PCR or qPCR. (B) Schematic of the signaling events that define preplacode at the late gastrula stage and the characteristic genes expressed by the different tissues. Epidermis, preplacodal ectoderm (PPE), neural crest (NC) and neural crest are specified along a high to low BMP gradient that is accompanied by FGF signaling. (C) Expression of endogenous BMP4 is detected in hESC and cells differentiated in DFBN. (D) Fold change of expression of the PPE genes SIX1 and GATA3, the otic placode gene PAX2 and the epidermis marker TP63 in the differentiated cells relative to hESC. Concentrations (in brackets) are given in ng/ml for

BMP4 and nM for LDN212854. (E) Normalized expression of the genes of interest with 0 being equivalent to hESC expression and 1 being the maximal expression across the differentiation conditions tested. (G) Immunocytochemistry shows that cells treated with 20 nM LDN212854 (LDN20) during differentiation express SIX1 and PAX2 protein (green). Nuclei were stained with Hoechst. (F) Quantitative analysis of the proportion of LDN20 cells found to be SIX or PAX2 positive. All error bars are given as standard error of the mean.

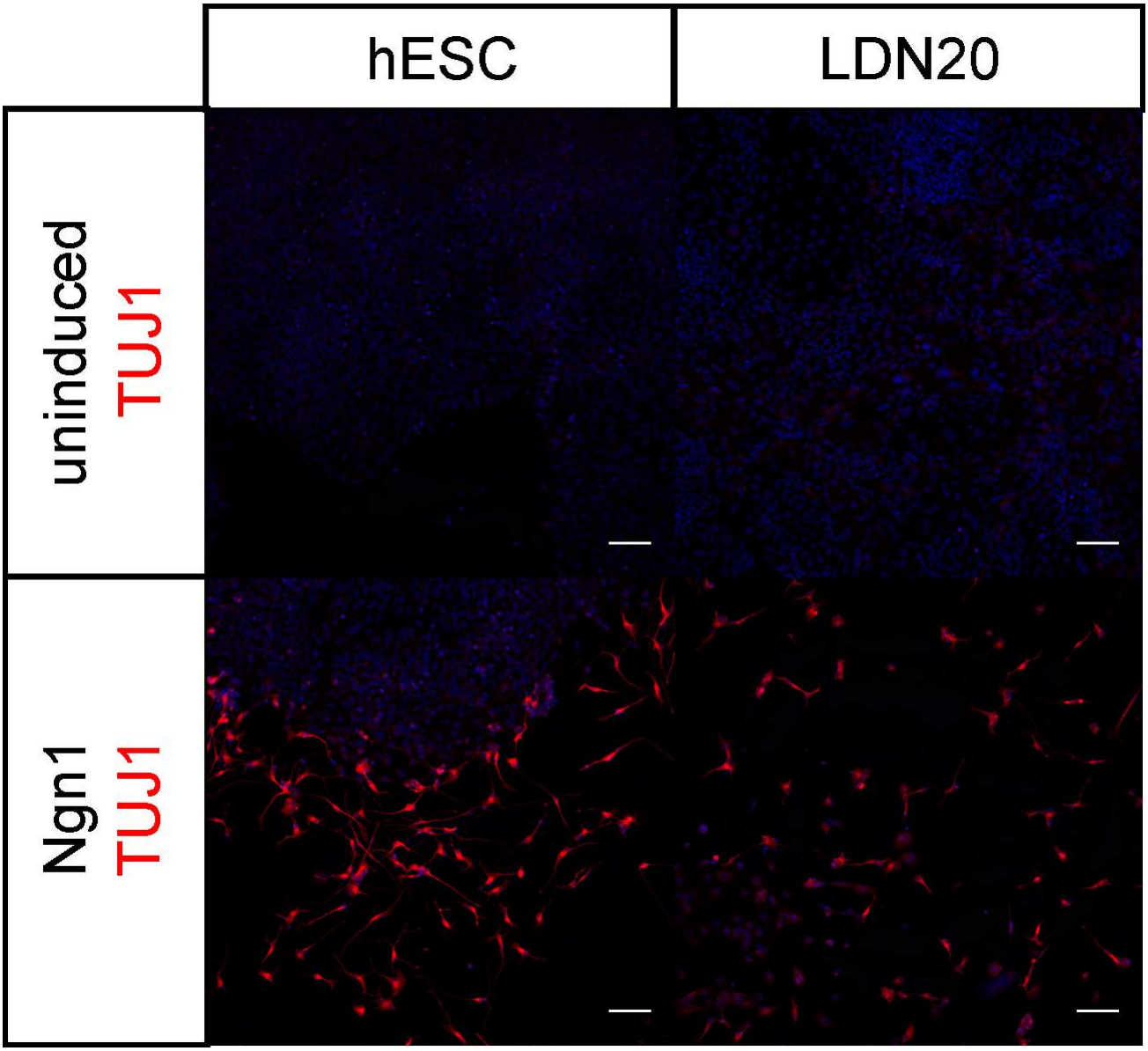

Figure 2: Adenoviral overexpression of Ngn1 in hESC and LDN20 cells. Immunohistochemistry 5 days after adenoviral transduction shows expression of the neuronal marker TUJ1 in hESC and LDN 20 cells. Untreated cells show no staining. Scale bars 100 µm.

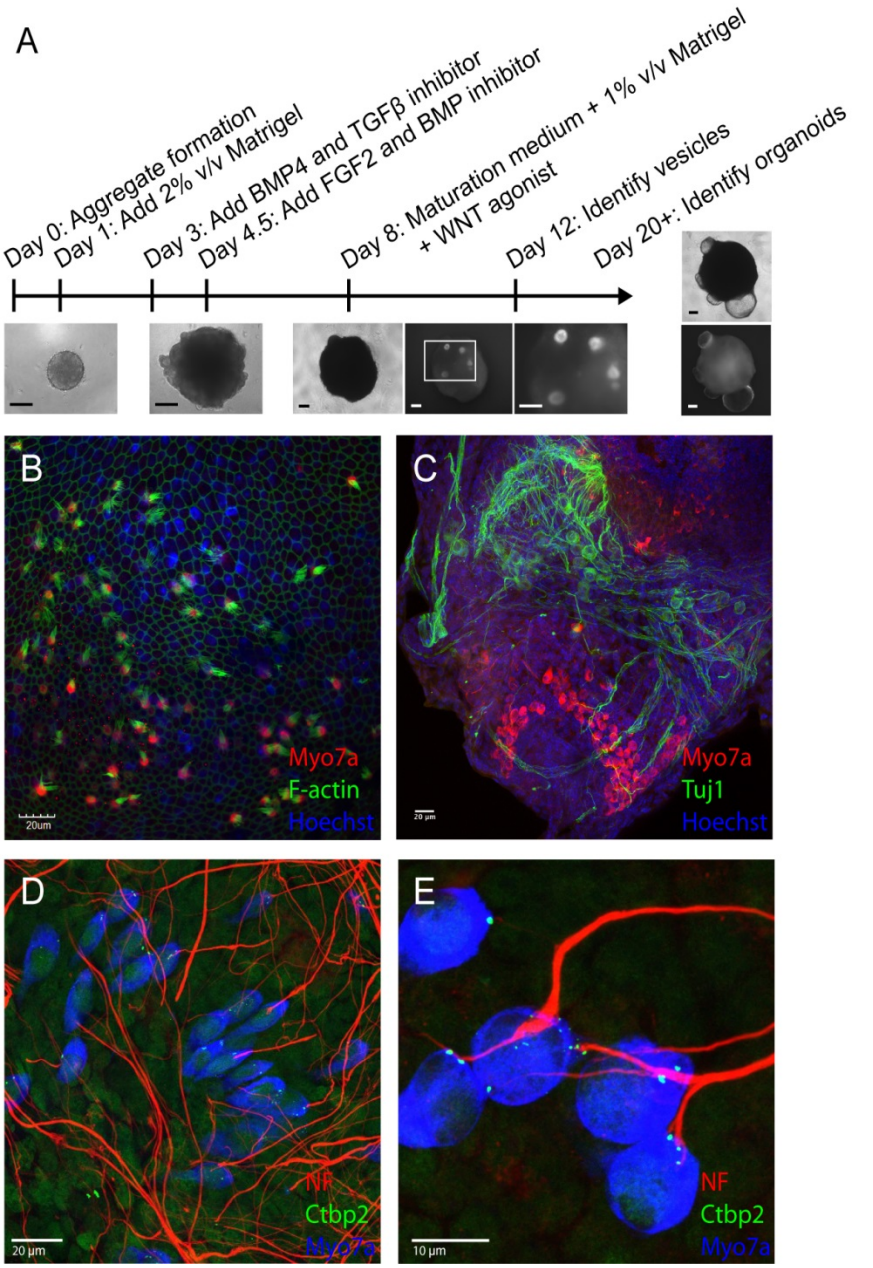

Figure 3: Generation of neurons within inner ear organoids. A: Outline of organoid culture protocol. Mouse embryonic stem cells (mESCs) are aggregated into embryoid bodies on day 0 and subsequently treated with a series of growth factors and small molecules to promote stepwise differentiation from mESCs to non-neural ectoderm to otic placode. After the aggregates are moved to maturation medium at day 8, $EGFP^{+}$ otic vesicle-like structures become visible under epifluorescence when a $Pax2^{EGFP/+}$ mESC line is used. By day 20, vesicles have moved to the outer surface and expanded; innervated hair cells are found at the interface (termed the "organoid") between the expanded vesicle and the rest of the aggregate. Scale bars = 200 µm. B-E: Confocal projections of organoid tissues dissected and stained via immunofluorescence. B: Hair cell markers Myo7a (red) and F-actin (green). C: Hair cell marker Myo7a (red) and neuron marker Tuj1 (green). D and E: Hair cell marker Myo7a (blue), presynaptic marker Ctbp2 (green), and neuron marker neurofilament (red). Ctbp2 puncta indicate the presence of ribbon synapses.

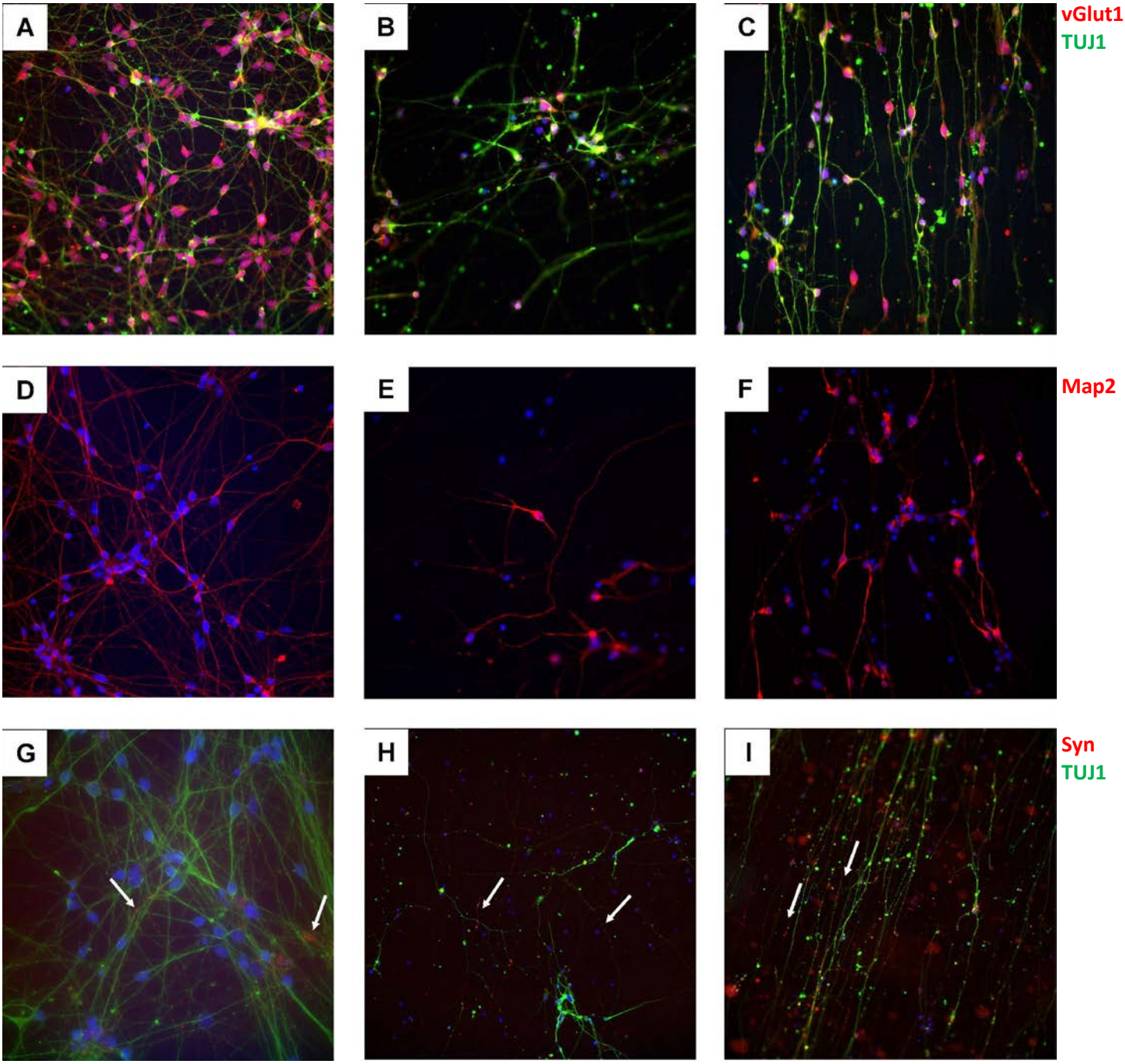

Figure 4: Consistency of neuronal fate on PCL nanofibers. Immunocytochemistry for neurotransmitter fate and terminal differentiation of neurons grown on polystyrene coverslips (A,D,G) or random (B,E,H) and aligned nanofibers (C,F,I) 4 weeks into terminal differentiation. All conditions are counterstained with Hoechst to visualize nuclei. Syn = synaptophysin

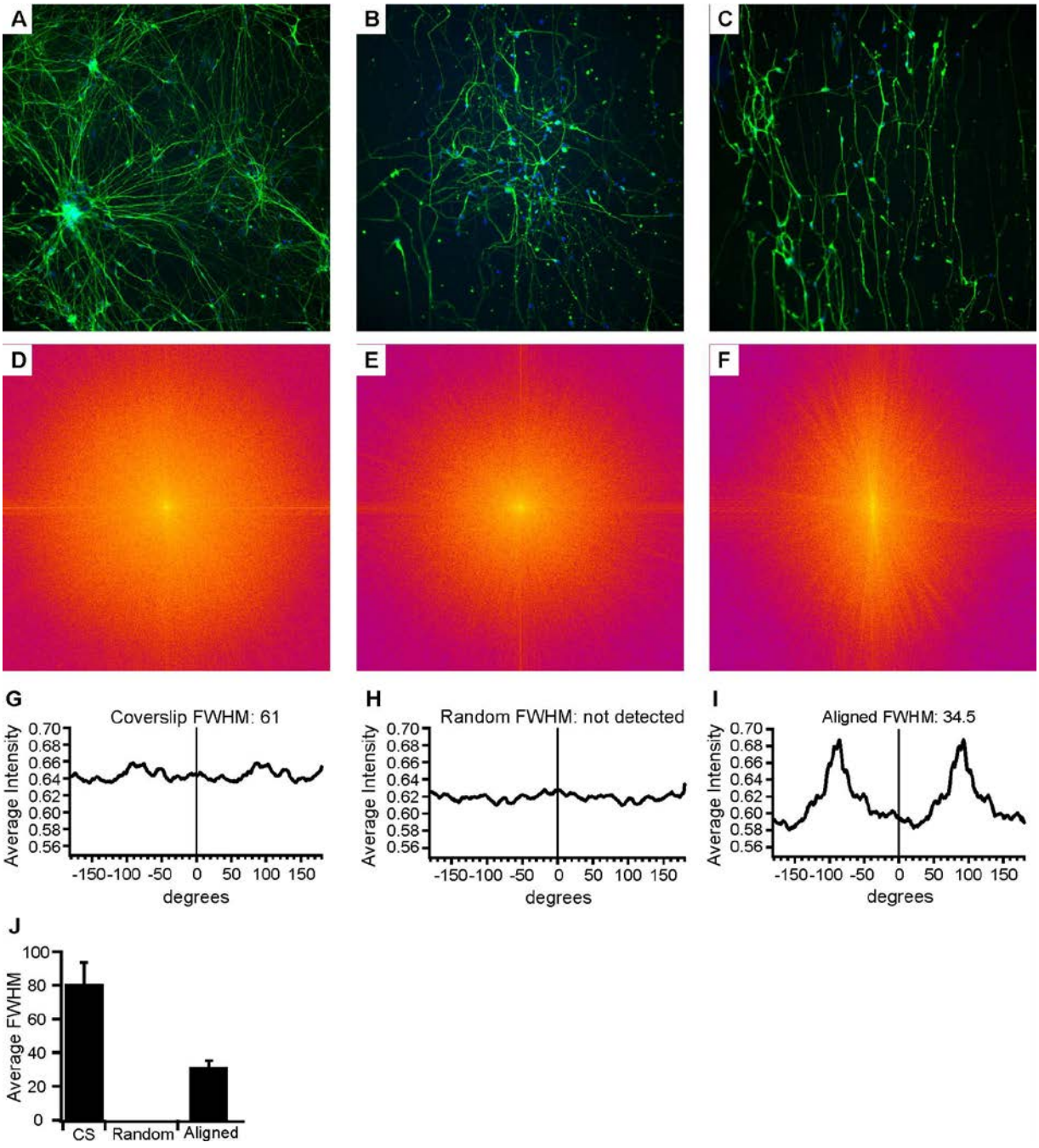

Figure 5. Alignment analysis of neurite growth. (A – C) Representative images of neurons grown on coverslips (A), random fibers (B) and aligned fibers (C) after 4 weeks of terminal differentiation (20x objective). (D – F) Fourier transformations (FFT) of the images shownin (A – C); (D coverslip, E random, F aligned). Yellow depicts greatest intensity, blue depicts least intensity. Note that narrower areas of higher intensity (yellow-orange) in the FFT images correlate with more oriented neurites. (G – I) Image intensities of (D – F) as a function of angle. (G) Average intensity of (D), (H) average intensity of (E), (I) average intensity of (F). For every condition the difference between angles on each side of the peak corresponds to one-half the peak height, full width-half max (FWHM) was calculated in order to quantify the alignment. Small FHWM values correspond to high alignment. (J) Average FWHM values of representative images derived from neurons grown on coverslips (n = 8) and aligned fibers (n = 6) for 2 - 6 weeks. For random fibers, only one out of 7 samples allowed calculation of FWHM.

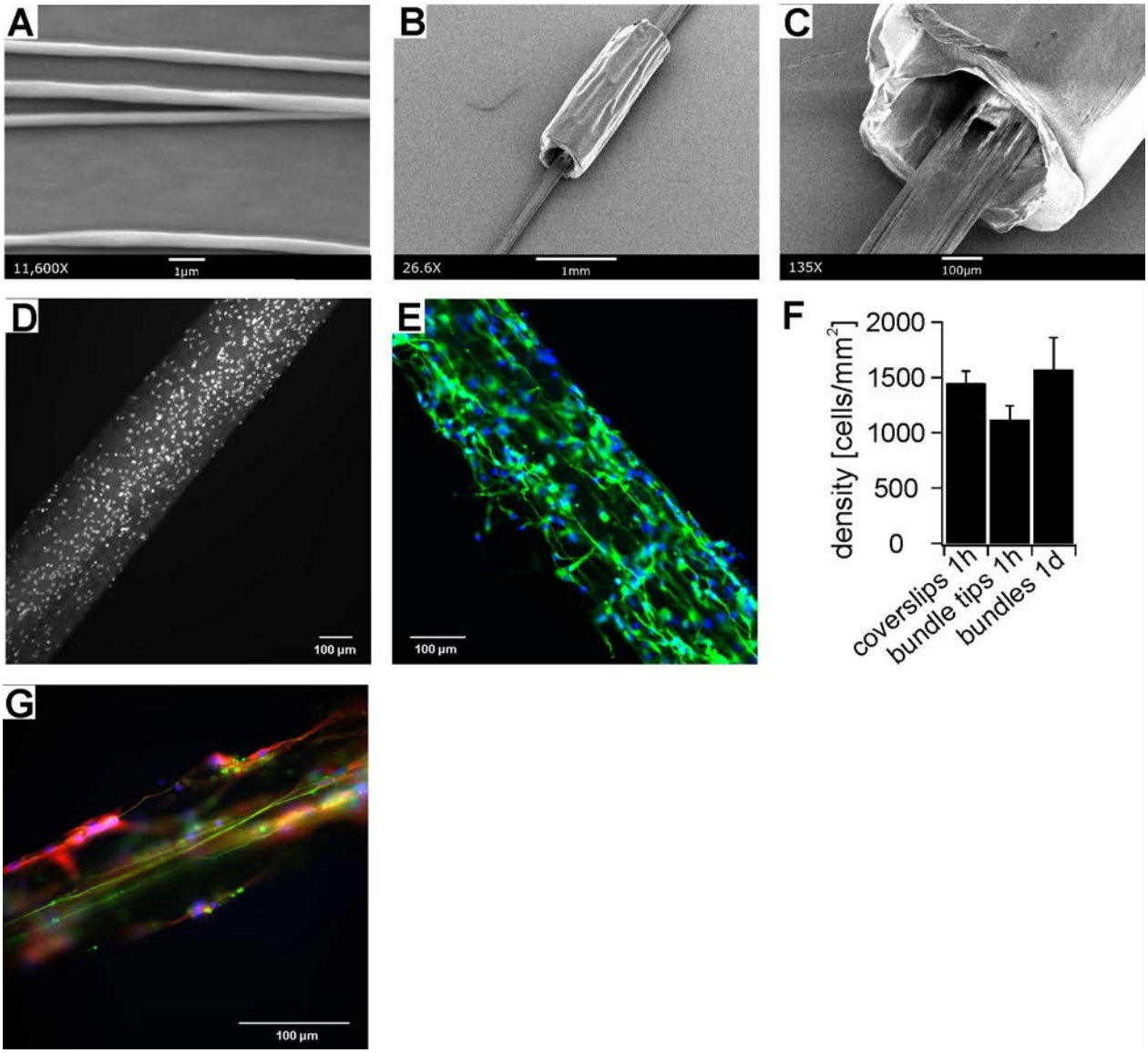

Figure 6. Nanofibers and scaffolds for implantation. (A-C) SEM images of nanofibers and scaffolds. (A) Typical fiber diameters are below 1 µm. (B) and (C) Conduits were designed to be 500 to 700 µm in diameter and 2 mm in length. A bundle of fibers is placed in the center of a polymer sheath. (D – G) Cell density and neurite outgrowth on seeded conduits. (D) Cell density visualized with Hoechst stain of nuclei on a fiber bundle 1 h after seeding. (E) Neurite outgrowth on bundles 1 day after seeding (TUJ1, green). (F) Comparison of cell density 1 h after seeding on coverslips and bundle tips and 1 day after seeding on bundles. (G) Neurite out growth on a fiber bundle after 6 weeks of culture within the polymer sheath. Cells were stained with the neuronal marker TUJ1 (green) and the neural linage marker Nestin (red). Nuclei are stained with Hoechst.

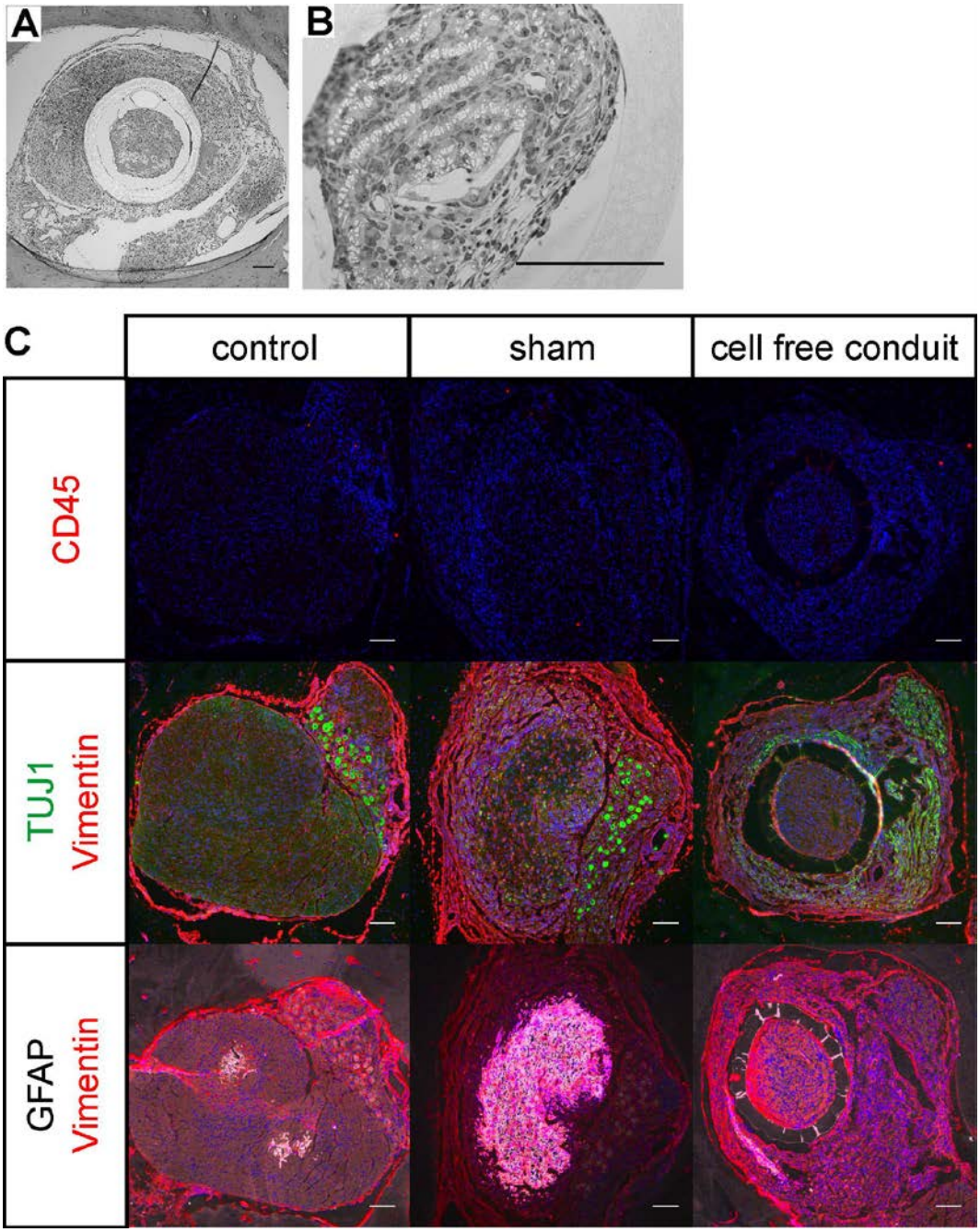

Figure 7. Tissue response to cell free conduit implantation. (A-B) Plastic sections of cell free conduits implanted into the IAM of guinea pigs. (A) Placement of conduits in the IAM. (B) Host tissue is invading the interior of the conduit and filling the spaces between nanofibers. Scale bars 100 µm. (C) Immunohistochemistry of untreated controls, sham operated and cell free conduit implanted guinea pig IAMs. For sham and cell free implants animals were deafened by ouabain injected through the round window two weeks prior to implantation surgery. Sections are negative for the hematopoietic marker CD45 indicating the absence of an inflammatory response. Positive controls for the CD45 antibody were performed by staining guinea pig blood cells (data not shown). Staining for the astroglia marker GFAP (white) and the Schwann cell/fibroblast marker vimentin (red) indicate that both cell types are involved in scar formation. However, cells inside the conduit stain positive for vimentin, indicating Schwann cell migration into the conduit. Scale bars **100** µm.

A

Stem Cells implanted on Study Day 16:

ID	D-25	D-35	D-44
DSC-133	375µA	320µA	260µA
DSC-137	300µA	340µA	320µA
DSC-138	180µA	130µA	100µA
DSC-139	360µA	200µA	-
DSC-140	210µA	170µA	-
DSC-143	-	-	-
DSC-144	-	-	-

Control Subjects implanted on Study Day 16:

ID	D-25	D-35	D-44
DSC-141	810µA	500µA	-
DSC-142	500µA	-	-
DSC-145	-	-	-
DSC-146	-	-	-

B

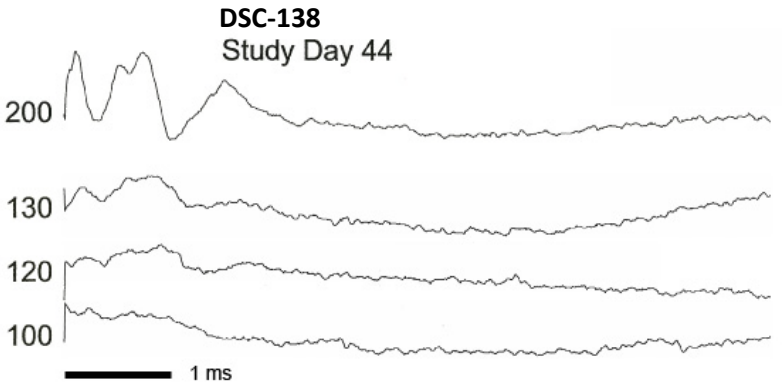

Figure 8. Functional recovery after implantation. (A) Electrical ABR thresholds for deafened guinea pigs implanted with nanofibrous scaffolds and neural progenitors using Approach 1 (see Objective 1 text) compared to deafened, implanted control subjects with cell-free scaffolds. Time points marked with a dash (-) represents data that is still forthcoming. (B) Example electrical ABR waveforms for cell-implanted subject DSC-138 28-days after implantation.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Robert Keith Duncan
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-8949-8727
Nearest person month worked:	8
Contribution to Project:	Dr. Duncan is an Associate Professor of Otolaryngology in the Kresge Hearing Research Institute. He is a physiologist with expertise in ion channel structure and function. As Principal Investigator, Dr. Duncan will supervise all aspects of the proposed studies including procedural design, data analysis and interpretation, and determination of new directions. He will prepare peer-reviewed manuscripts and present the results at national meetings. Dr. Duncan will also actively participate in collection of electrophysiology data. He will coordinate experiments with collaborating laboratories (Drs. Corey, Miller, and Shore) and core facilities (Drs. Lyons and Cavalcoli). Dr. Duncan will ensure close interaction of the investigating team through weekly project meetings.
Funding Support:	New Award: Sponsor: Hide and Seek Foundation / SOAR Title: Mitigation of Cyclodextrin Ototoxicity Award Period: 09/01/15 – 09/30/16 Effort: 0.0 calendar months Award Amount: $113,636

Name:	Dr. Joseph Corey, M.D., Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	3
Contribution to Project:	Dr. Corey is an expert in using patterned substrates to guide the outgrowth of primary neurons in culture. He will supervise the construction of aligned nanofiber substrates and the covalent linkage of bioactive molecules, if required. Dr. Corey has a joint UM-VA appointment. A memorandum of understanding is on file. All effort will come from Dr. Corey's UM appointment.
Funding Support:	New Award: Sponsor: VA RR&D Merit Review, PI: Corey JM Award Period: 10/01/2015 – 09/30/2017 Title: Cell-Integrated Microfibers for Improved Nerve Regeneration Ended Awards: Sponsor: VA RR&D Merit Review, , PI: Corey JM Award Period: 04/01/2010 – 09/30/2015 Title: Surface Modified Nanofibers for Nerve Regeneration

Name:	Dr. Josef Miller, Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to Project:	Dr. Miller is an expert in cochlear physiology, drug delivery, and the prevention of hearing loss. His laboratory has extensive expertise in surgical approaches to place electrode arrays and perfusion devices into the cochlear duct. He will directly supervise Ms. Prieskorn, a member of his laboratory staff, in placement of the nanofiber array in guinea pigs, installation of a stimulating electrode, and measurement of electrically evoked auditory brainstem responses.
Funding Support:	No Change

Name:	Dr. Susan Shore, Ph.D.
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to Project:	Dr. Shore is an expert in the physiology of the auditory brainstem. She has significant expertise in tract tracing, preparation of cochlear nucleus slices, and multielectrode recordings of electrically and acoustically evoked activity in the cochlear nucleus. Dr. Shore will assist in evaluating integration of hiPS-derived neurons in cochlear nucleus explants and *in vivo* preparations. She will also guide electrophysiology experiments, recording CN responses from electrical stimulation of the hiPS-seeded nanofiber array implanted into guinea pigs.
Funding Support:	Funding Extended: PI: Shore, Award Period: 07/01/14 – 03/31/16 Sponsor: UM Coulter Translational Research Partnership Program Effort: 0.5 calendar months Award Amount: $140,327 year Title: Combined Auditory-Somatosensory Stimulation to Alleviate Tinnitus

Name:	Liqian Liu
Project Role:	Research Technician Associate
Researcher Identifier (e.g. ORCID ID):	Not Applicable
Nearest person month worked:	12
Contribution to Project:	Ms. Liu is an exceptional molecular biologist with expertise in cell culture, stem cell differentiation, quantification of gene expression, preparation of cloned gene products, and protein analysis (Western blot, co-immunoprecipitation, sucrose gradient fractionation). Ms. Liu will be responsible for maintaining hiPS cell stocks, active cultures, differentiation into neural cells, and cell sorting. She will also prepare RNA for gene expression studies and perform histology experiments as needed. Ms. Liu manages animal colonies, reagent inventory, and all purchasing.
Funding Support:	NA

Name:	Sandra Hackelberg
Project Role:	Post-Doctoral Fellow
Nearest person month worked:	12
Contribution to Project:	Dr. Hackelberg will be responsible for designing, conducting, and analyzing electrophysiology experiments. Sandra will work with Ms. Prieskorn to implant guinea pigs and perform all electrophysiological / histological experiments. She also will be responsible for manuscript preparation and scientific communication at national meetings.
Funding Support:	NA

Name:	Diane Prieskorn
Project Role:	Admin Specialist Senior Health
Researcher Identifier (e.g. ORCID ID):	Not Applicable
Nearest person month worked:	3
Contribution to Project:	Ms. Prieskorn is an expert in surgical approaches to the guinea pig cochlea, including chronic placement of electrical arrays and drug delivery apparatus'. She will work closely with Drs. Miller and Purcell to place nanofiber arrays into the internal auditory meatus and evaluate functional integration with electrically evoked auditory brainstem responses.
Funding Support:	NA